Sugar Tech (2008) 10(1) : 91-98

RESEARCH ARTICLE

Recent advances in functional genomics for sugar beet (*Beta vulgaris* L.) improvement: progress in determining the role of *BvSTI* in pest resistance in roots

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Received: 14 December 2007 / Accepted: 20 January 2008

Abstract To gain knowledge of root resistance mechanisms in sugar beet, *Beta vulgaris* L., our laboratory has been studying the interaction of sugar beet with its most devastating insect pest, the sugar beet root maggot (SBRM; *Tetanops myopaeformis* Roder). Damage from SBRM infestations is a serious problem and current control measures rely on environmentally damaging insecticides. We recently reported root-specific gene expression incited by SBRM feeding in a moderately resistant F1016 and a susceptible parental F1010 line. A cDNA expressed sequence tag (EST) coding for a serine (trypsin-type) protease inhibitor (*BvSTI*) was identified and investigated further here. *BvSTI* shares sequence similarity with a root-specific tomato gene whose expression is induced by insect feeding. Since serine proteases comprise the major digestive enzymes in root maggot midguts, we hypothesize *BvSTI* may be involved in resistance. To elucidate the functional role of *BvSTI*, its coding region was fused to the CaMV 35S promoter and constitutively expressed in sugar beet hairy roots and *N. benthamiana* plants. In *BvSTI*-transformed F1010 hairy roots, trypsin inhibitory activity increased 2 to 4-fold. Using a polyacrylamide gel assay, new trypsin-like PI activity was detected in *BvSTI-N. benthamiana* plants. Since SBRM cannot be reared *in vitro*, two other insects that utilize serine digestive proteases, fall armyworm (*Spodoptera frugiperda*) and tobacco hornworm (*Manduca sexta*), were screened for resistance. To date, we demonstrated that 1) fall armyworm will feed on sugar beet hairy roots and 2) tobacco hornworm fed *BvSTI-N. benthamiana* leaves had reduced weights and pupal sizes. These results suggest that *BvSTI* may contribute to the moderate resistance of F1016 roots to SBRM. Functional analysis of additional ESTs will further support efforts to characterize the components of sugar beet root resistance mechanisms.

Keywords Sugarbeet, root, functional genomics, *BvSTI* gene, pest resistance

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Introduction

Introgression of resistance genes in sugar beet (*Beta vulgaris* L.) through traditional breeding has been challenging, especially when gene transfer is attempted between annual plants or wild relatives and the cultivated biennial plants. Very often conventional breeding attempts are plagued by deleterious effects on yield and sucrose content (Schulte *et al.,* 2006). Use of biotechnological approaches for genetic modification overcomes some of the constraints and difficulties associated with conventional breeding and accelerates the development of better adapted hybrids for improved agronomic productivity. Biotechnology facilitates the direct transfer of genes from other *Beta* species without the introduction of undesirable traits, as well as genes from unrelated organisms that can be utilized for sugar beet improvement (Cai *et al*., 1997). Recent advances in sugar beet transformation technology are improving the overall speed with which genetically modified sugar beet plants can be produced (for review, see Smigocki *et al*. 2007b; Hall *et al*., 1996; Hisano *et al*., 2004; Ivic-Haymes and Smigocki, 2005a, b; Snyder *et al*., 1999). The success of genetic modification

of sugar beet for resistance to broad-spectrum herbicides, beet necrotic yellow vein virus and *Cercospora* leaf spot disease have been well-documented (De Block *et al.* 1987; Kishchenko *et al.* 2005; Lathouwers *et al*. 2005; Lennefors *et al*. 2006; Maleki *et al*. 2003; Mannerlöf *et al*. 1996, 1997; Pidgeon *et al*. 2005; Tertivanidis *et al.* 2004). Recent deregulation of genetically modified, herbicide resistant sugar beet for commercial production in the United States underscores the success of biotechnological approaches for sugar beet improvement.

Great efforts are being made to identify novel sources of resistance in wild relatives and cultivated varieties for use in sugar beet improvement programs (Francis and Luterbacher 2003; Panella and Lewellen, 2006). A small number of studies are also focusing on the identification of sugar beet genes that are expressed in resistant or susceptible interactions with pests or pathogens (Herwig *et al*., 2002; Kloos *et al*., 2002; Larson *et al*., 2007; Nagendran and McGrath 2006; Nielsen *et al.,* 1997; Puthoff and Smigocki 2007; The Institute for Genomic Research, Rockville, MD). Such studies are anticipated to lead to the discovery of sugar beet genes that function in resistance mechanisms and to facilitate the comparison of resistance responses to several pests and pathogens.

To gain knowledge of root defense mechanisms, our laboratory has been studying the interaction of sugar beet with the sugar beet root maggot (SBRM; *Tetanops myopaeformis* Roder) (Puthoff and Smigocki, 2007; Smigocki *et al*., 2006). Root damage from SBRM feeding is a serious problem in the U.S. and Canada and leads to significant reductions in sugar yields. The insect affects approximately 50% of the more than 0.6 million hectares grown in the U.S. Without control measures, yield losses in the Red River Valley would commonly reach about 40%. Destruction of lateral roots and, under severe infestations, complete severing of the main taproot has been observed (Dregseth *et al*., 2003). Control of SBRM, as with other major pests, continues to rely on environmentally damaging insecticides since germplasm with complete resistance is not available. Most recently, two sugar beet lines (F1015 and F1016) with a moderate level of genetic resistance have been released for use in sugar beet breeding programs (Campbell *et al*., 2000). The F1016 germplasm provides the highest reduction (approximately 40%) in SBRM damage ratings under field conditions. To identify root genes responsible for this resistance, we profiled root genes that are modulated by SBRM feeding in the moderately resistant F1016 and the susceptible F1010 line (Puthoff and Smigocki, 2007). More than 160 genes were identified using suppressive subtractive hybridization as an enrichment method for genes that respond to SBRM feeding. Approximately 80% of randomly chosen F1016 cDNA expressed sequence tag (EST) clones showed differential hybridization that was confirmed by RT-PCR analysis (Puthoff and Smigocki, 2007). The largest number of identified ESTs from both the susceptible and resistant genotype grouped into the defense and stress response classes. These include polyphenol oxidase, beta-glucosidase, glutathione S transferase and subtilisin-like serine protease. In addition, PR protein genes (chitinase, oxalate oxidase-like, peroxidase, PR10-like) typically induced after microbe infections were also found to be regulated by SBRM feeding. No characteristic wound response genes such as lipoxygenases or phenylalanine ammonia lyases were found except for one proteinase inhibitor (PI) clone. The PI clone, *BvSTI,* encodes a protein with a conserved motif denoting it as a member of the Kunitz trypsin-type (serine) proteinase inhibitor family (Jofuku and Goldberg, 1989). A smaller group of genes involved in secondary metabolism and signal transduction were also identified among the selected ESTs. Gene expression profiles of the cloned ESTs were obtained using macroarrays following mechanical wounding and treatment of roots with methyl jasmonate, salicylic acid and ethylene. Of the examined root ESTs, the greatest number were regulated by methyl jasmonate and salicylic acid, suggesting these signaling pathways may be involved in sugar beet root defense responses to SBRM. The results from this study have allowed us to formulate testable hypotheses as to the function of several of the cloned root genes in insect resistance mechanisms.

We are currently working on the functional analysis of one of the identified clones, the *BvSTI* gene. *BvSTI* shares sequence similarity with the tomato *LeMir* gene that is primarily expressed in the maturing epidermis of the tomato root, is induced by invading nematodes and the protein is secreted to the rhizosphere (Brenner *et al*., 1998). We previously determined that serine and aspartyl proteases are the major digestive enzymes in SBRM midguts and that their activity is effectively blocked by soybean trypsin-chymotrypsin and squash aspartyl proteinase inhibitors, respectively (Wilhite *et al*., 2000). Therefore, our findings suggest that the moderately resistant F1016 germplasm may incorporate the *BvSTI* gene in its defense arsenal against SBRM by forming a zone of protection surrounding the moderately resistant roots and acting as a first line of defense in the peripheral cell layers. We report here our progress on characterization of the *BvSTI* gene and its over-expression in sugar beet hairy roots and *N. benthamiana* plants for analysis of its role in insect resistance and root biology.

Materials and Methods

Plant material

Sugar beet breeding lines F1010 (Campbell, 1992) and F1016 (Campbell *et al*., 2000) and *N. benthamiana* plants were grown in the greenhouse at 20 to 30°C during the day and 18 to 25°C at night with an approximate day length of 14 to 16 h.

BvSTI gene constructs

To obtain the full length coding sequence of *BvSTI* from the cloned EST, 5' and 3' RACE kits (BD Biosciences, San Jose, CA) were used with gene specific primers. After both 5' and 3' RACE clones were sequenced, gene specific primers were designed and used to amplify the entire *BvSTI* coding region (Smigocki *et al*., 2007a). *BvSTI* was fused to the 35S promoter in the binary plasmid pCAMBIA 1301 plant transformation vector (pBvSTI) that carries the HPTII selectable marker gene for selection of hygromycin (Hg) resistant, transformed cells (CAMBIA, Canberra, Australia).

Plant transformation

Agrobacterium rhizogenes strain 15834 carrying the pBvSTI transformation vector was grown overnight and then resuspended in liquid half strength (1⁄2) B5 medium (Gamborg *et al*., 1968). Sugar beet petiole transformation was essentially done as described by Kifle *et al*. (1999). Petioles were excised from fully expanded leaves of greenhouse-grown plants and surface-sterilized. Petioles were then cut into 1 cm segments and infected with the *A. rhizogenes* strains for 10 min, blotted dry and plated on 1/2 B5 medium. After 2d of co-cultivation in the dark at 25^0C, explants were washed with Cefotaxime and carbenicillin (500 mg/l each) and plated on 1⁄2 B5 medium containing 250 mg/l of each of the above antibiotics. Regenerated hairy roots were excised and cultured on 1⁄2 B5 medium containing 5 mg Hg sulfate/l.

N. benthamiana leaf disks were transformed with *A. tumefaciens* strain EHA105 carrying the pBvSTI gene construct as previously described (Smigocki, 1991; Smigocki *et al*., 2007a). Transformants were selected on 20 mg Hg sulfate/l and regenerated shoots were rooted on Murashige and Skoog media with B5 vitamins and 20 mg Hg sulfate/l. Plants were transferred to the greenhouse and fertilized twice a month with Peters 20:20:20.

RT-PCR analysis

Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen). Titanium One-Step RT-PCR Kit (Clontech Laboratories) was used to amplify the BvSTI transgene transcripts using 100 ng of total RNA.

BvSTI proteinase inhibitor activity assays

Soluble proteins were extracted in 1 mM HCl and analyzed using a radial diffusion activity assay (Broadway and Missurelli, 1990; Jongsma *et al*., 1993). Proteins were quantified (Bradford, 1976; Bio-Rad) and immediately used in the assay. Samples were added to wells (4 mm in diameter) imprinted in agarose gels that contained 0.001 mg bovine trypsin/ml (Sigma) as substrate. After incubation at 37°C for 30 min, digested trypsin was detected with N-acetyl-DL-phenylalanine ß-naphthyl ester (APNE; 2.5 mg/ml, Sigma) substrate and tetrazotized O-dianisidine dye solution (0.5 mg/ml, Sigma). Diameters of clear zones were measured around each well and PI activity quantified using soybean trypsin PI (Fluka) standard curve. For further analysis of trypsin proteinase activity, native proteins were extracted in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM EDTA, 10% sucrose, 10 mM ascorbic acid, 1 mM PMSF, 2 mM DTT (Chan and De Lumex, 1982; Wang *et al*., 2003). Proteins (5-10 µg) were separated on 12% native polyacrylamide gels and after removal of SDS, proteins were re-natured and incubated with the bovine trypsin substrate. Acetic acid (10% v/v) was added to stop the reaction and clear zones corresponding to trypsin inhibitory activity were recorded.

Insect assays

In vitro propagated hairy roots were used to develop a fall armyworm (*Spodoptera frugiperda*) feeding assay. Fall armyworm eggs were incubated on sugar beet leaves for hatching. Newly hatched larvae were transferred to Petri dishes containing 14 day old hairy roots on water-moistened filter paper. Degree of larval feeding and weights were recorded daily.

Third instar tobacco hornworm (*Manduca sexta*) larvae were fed *N. benthamiana* leaves from the *BvSTI* transformed or the untransformed control plants. Larval sizes were recorded until pupation.

Results

BvSTI gene expression

A number of independently transformed sugar beet hairy root lines carrying the *BvSTI* gene were regenerated from both the SBRM susceptible F1010 and moderately resistant F1016 petioles (Fig. 1A). Hairy root cultures displayed a varied pattern of root growth, which was relatively slow, moderate

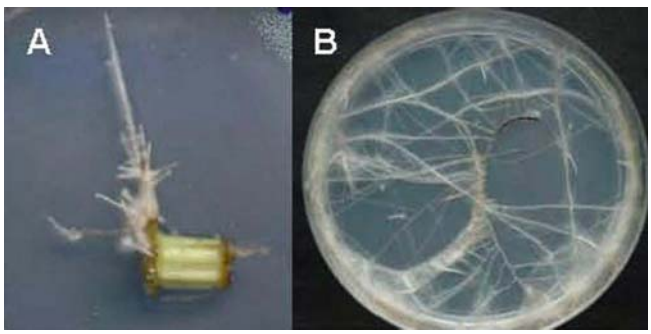

Fig. 1. A) Hairy roots regenerating from a sugar beet petiole (F1016). B) Excised F1016 hairy roots transformed with *BvSTI* after 3 weeks in culture.

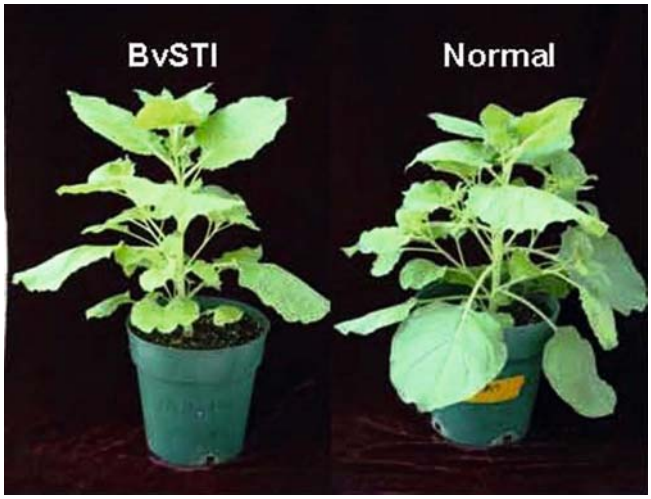

Fig. 2. *N. benthamiana* plants transformed with the *BvSTI* gene (left) as compared to normal, untransformed (right) plants.

(Fig. 1B) or rapid. Preliminary analyses indicated that the rate of hairy root growth was not inversely proportional to the level of *BvSTI* gene expression, i.e. slow growth, high levels of expression (data not shown). *N. benthamiana* plants transformed with the *BvSTI* gene exhibited phenotypes that were indistinguishable from the normal, untransformed plants (Fig. 2).

Analysis of *BvSTI* transcript levels in the F1010 hairy root cultures revealed elevated transcript levels of the introduced gene in the transformants (Fig. 3, Lanes 1-4). Low levels of *BvSTI* transcripts were detected in the F1010 control transformed with the empty vector (C), therefore, we speculate that it may be the regulation of *BvSTI* expression that may contribute to SBRM resistance in F1016. The *N. benthamiana BvSTI* transformed plants showed a high level of transcription of the introduced sugar beet gene that was not detected in the untransformed control plants (Fig. 3B).

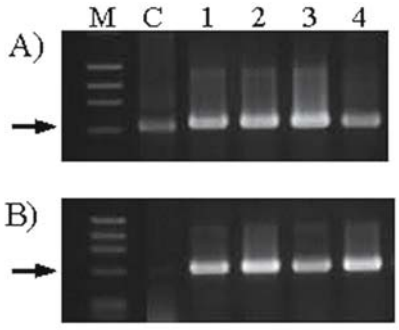

Fig. 3. RT-PCR analysis of *BvSTI* gene expression in transformed (A) sugar beet hairy roots and (B) *N. benthamiana* plants. M, Lambda HindIII molecular weight markers; C, untransformed control; arrow, 0.6 kb.

BvSTI Activity

A radial diffusion assay was used for analysis of *BvSTI* PI activity in transformed plant tissues. The radial diffusion method allows for rapid analyses of large numbers of transformants as an initial screen for levels of recombinant PI protein activity in small quantities (100 mg) of transformed plant tissues. Analysis of PI activity in five independently derived F1010 sugar beet hairy roots transformed with the *BvSTI* gene revealed elevated levels of PI activity (Fig. 4, samples 1 and 3-6) that were not observed in controls transformed with the empty vector (Fig. 4, sample 2C). PI activities ranged from about 0.04 to almost 0.07 µg equivalent/ µg protein in the *BvSTI* lines as compared to 0.02 µg in the empty pCAMBIA 1301 vector control.

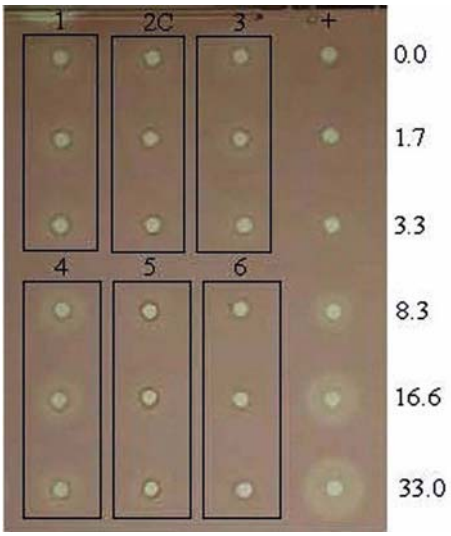

Fig. 4. Trypsin inhibitor activity detected in *BvSTI* transformed sugar beet hairy roots using a radial diffusion agarose gel assay. Boxes 1 and 3- 6 represent five independent transformants, in triplicate; Box 2 is a negative, vector-transformed control. +, soybean Kunitz trypsin inhibitor protein as positive control at 0, 1.7, 3.3, 8.3, 16.6 and 33.0 µmoles.

Transformants initially determined to have higher PI activities were further analyzed on PAGE zymograms to identify the *BvSTI* transgene product. Two independently derived F1010 sugar beet hairy root lines (Fig. 5A, Lane 1-4) and four *N. benthamiana* plants (Fig. 5B, Lane 1-4) transformed with the *BvSTI* gene revealed new activity bands (i.e. clear zones) that likely correspond to the introduced gene product. As expected, other protein bands corresponding to endogenous PI activities were also observed in both the sugar

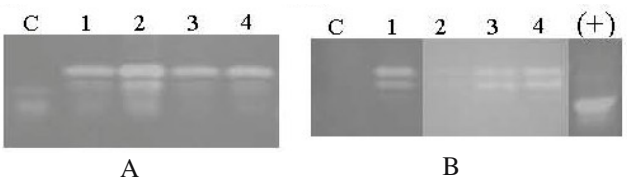

Fig. 5. Trypsin inhibitor activity detected in *BvSTI* transformed (A) F1010 sugar beet hairy roots; lane 1-2, transformant 202 and lane 3-4, 207. Total protein: 5 µg in lane 1 and 3 and 10 µg in lane 2 and 4. (B) *N. benthamiana BvSTI* transformants 1-4 (5 µg total protein per lane). C, untransformed controls (5 µg); +, soybean Kunitz trypsin inhibitor, 20 kDa, 1.5 µg.

beet hairy roots and *N. benthamiana* plants. Presence of the recombinant protein in the transformed tissues will be confirmed by Western blot analysis using polyclonal antibodies prepared from a mixture of the two most antigenic peptides of BvSTI (GenScript Corp.; Smigocki, unpublished)).

Insect Assays

BvSTI transformed hairy root lines showing high levels of proteinase inhibitor activity will be used in *in vitro* bioassays to determine the effect of the *BvSTI* gene on larval feeding. We previously demonstrated a preference of first-instar SBRM for the susceptible F1010 germplasm as compared to the moderately resistant F1016 hairy roots (Smigocki *et al*., 2006). We observed distinct larval behavior and feeding patterns on the susceptible vs. resistant line. These findings will aid the screening of *BvSTI* transformed hairy roots for resistance to SBRM larvae that are only available during the growing season from infested fields. Another pest of sugar beet that can be reared in the laboratory and is known to utilize serine proteases for digestion is the fall armyworm (Srinivasan *et al*., 2006). In this study, we demonstrated for the first time that fall armyworm will feed on sugar beet hairy roots (Fig. 6A) and thus can be used in insect bioassays to evaluate the effect of the *BvSTI* gene on resistance.

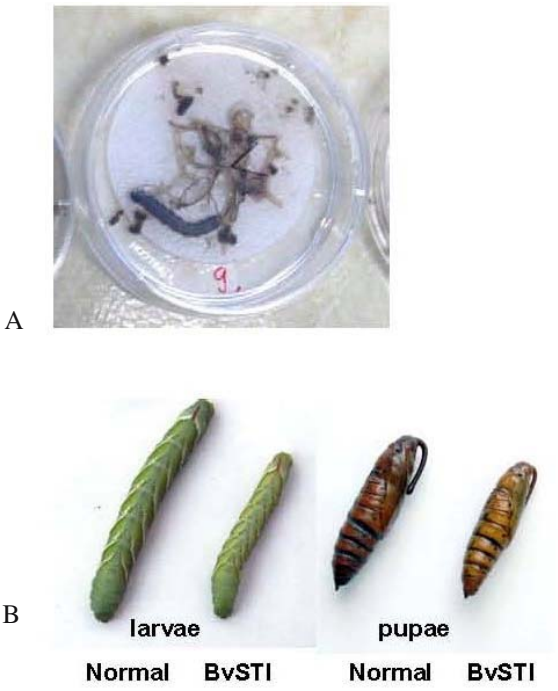

Fig. 6. (A) Fall armyworm (*S. frugiperda*) larvae feeding on *BvSTI* transformed F1010 sugar beet hairy roots. (B) Tobacco hornworm (*M. sexta*) larvae (left) and pupae (right) after feeding on *N. benthamiana* transformed with the *BvSTI* gene (*BvSTI*) or on normal, untransformed control (normal) plants.

Similarly, when tobacco hornworm larvae, insects well-known to utilize serine proteases for digestion (Srinivasan *et al*., 2006), were fed leaves of *BvSTI* transgenic *N. benthamiana* plants, we observed a substantial reduction in the overall sizes of the larvae and pupae (Fig. 6B). These preliminary findings will be validated in subsequent experiments with the T2 generation of *BvSTI* homozygous plants.

Discussion

To characterize genes involved in sugar beet root defense responses, we determined the profile of sugar beet root genes that were modulated by SBRM feeding in a moderately resistant F1016 line (Puthoff and Smigocki 2007). Of the more than 160 genes that were identified, an EST of particular interest that codes for a trypsin (serine) proteinase inhibitor (*BvSTI*) was reconstructed for functional analysis in plant cells. *BvSTI* was over-expressed in sugar beet hairy roots and *N. benthamiana* plants in order to characterize its potential role in SBRM resistance mechanisms.

The advantage of hairy root cultures is that they provide a rapid means (generally less than three weeks) for obtaining transformed sugar beet tissues that express foreign genes of interest. This approach provides for a rapid functional analysis of gene function *in planta*. Generation of transgenic plants that express the most interesting genes will still require the traditional labor-intensive and time-consuming transformation approaches as sugar beet hairy roots have proven to be recalcitrant to regeneration (Cai *et al*., 1997; Ehlers *et al*., 1991; Mannerlöf *et al*., 1996).

A number of studies investigating agricultural problems associated with sugar beet production have benefited from the use of sugar beet hairy roots. Mugnier (1987) examined the infection of transformed roots of sugar beet and table beet by *Polymyxa betae* and of brassicas by *Plasmodiophora brassicae*. He was able to identify two distinct fungal phases in the infected hairy root cultures. In addition, the culture of these obligate parasites on transformed roots allowed for detailed life cycle descriptions not previously obtainable under auxenic conditions. Similarly, experiments with *Heterodera schachtii* indicated that the larvae were capable of forming new cysts without loss of pathogenicity when cultured on sugar beet hairy roots (Paul *et al*., 1987). An *in vitro* approach was also used to demonstrate resistance to *H. schachtii* in a modified nematode-resistant sugar beet line, AN 5 (Paul *et al*., 1990). Detailed observations of SBRM larval feeding behavior and evaluation of the influence of bacterial additions on larval development were similarly assessed using axenic hairy root cultures (Smigocki *et al*., 2006; Wozniak, 1993). In the same way, these cultures were used to test the effect of toxic *N. tabacum* extracts on growth and development of SBRM larvae (Smigocki *et al*., 2003).

Since the insect cannot be reared in the laboratory, the

availability of SBRM larvae for bioassay experiments is limited to eggs and larvae collected from infested fields during the summer months (Smigocki *et al*. 2006). This creates a major delay in the bioassay of hairy root cultures transformed with putative SBRM resistance genes. Therefore, in this study, the hairy root cultures were used to develop bioassays for sugar beet pests other than SBRM. The fall armyworm is a pest that feeds on sugar beet foliage and is well-known to utilize serine protease enzymes to digest consumed plant material (Srinivasan *et al*., 2006). We demonstrate here that the fall armyworm will feed on sugar beet hairy roots *in vitro* (Fig. 6A). Based on these findings, experiments are in progress to analyze insect behaviour, quantity of plant tissue consumed and insect weight in order to assess *BvSTI* effectiveness in controlling the growth and survival of the fall armyworm. We expect to see reduced larval weights and/or mortality when the insects feed on the transformed tissues. However, the amount of tissue consumed may be greater or less than the amount consumed from the untransformed or empty vector controls. If the *BvSTI* gene inhibits digestion, insects may need to consume more tissue to obtain the nutrients needed to sustain growth and development (Ninkovic *et al*., 2007). In contrast, the presence of the inhibitors or enhanced defense responses may be a feeding deterrent resulting in less tissue consumed. Choice feeding tests may separate increased feeding from deterrent effects. Similar experiments are planned with the beet armyworm (*Spodoptera exigua*) and sugar beet root aphid (*Pemphigus populivenae*) once we establish that these insects will feed on sugar beet hairy roots.

To broaden the scope of potential target insects that utilize serine proteases for digestion, the *BvSTI* gene was over-expressed in a heterologous plant system. Transgenic *N. benthamiana* plants that express the *BvSTI* gene were fed to tobacco hornworm larvae (Srinivasan *et al*., 2006). Initial results revealed a substantial reduction in larval and pupal sizes (Fig. 6B). In order to confirm the results and test for resistance to other Lepidopteran insects (fall and beet armyworm, tobacco budworm), T2 generation of *BvSTI* transgenic plants are currently being selected for use in insect bioassay experiments. Although the results from this study are preliminary, they suggest that the serine proteinase inhibitor encoded by the sugar beet *BvSTI* gene may very well contribute to the resistance mechanism(s) in the moderately resistant F1016 roots and leads to increased SBRM tolerance.

Conclusions

The demand for well-characterized resistance genes is anticipated to intensify with the advent of biotechnological advances that will lead to increased worldwide cultivation of sugar beet. Production will increase to meet the demand for sugar and for other products that can be expected to be produced in sugar beet (Lathouwers *et al*., 2005; Skaracis, 2005). Recent examples of value added products that were produced in sugar beet include fructan, a desirable low calorie sweetener alternative, synthesized by genetic engineering with a heterologous gene encoding 1-sucrose:sucrose fructosyl transferase (Sevenier *et al*., 1998; Weyens *et al*., 2004). The development of a highly efficient process for converting sugar into an organic compound (hydroxymethylfurfural; HMF) that is used to manufacture polyester and diesel-like fuel should likewise make sugar beet a more profitable crop to farm (Zhao *et al*., 2007). Similarly, cultivation is expected to increase with the recent development of sugar beet varieties adapted for growth in traditionally unsuitable environments such as high-salt soils in China and tropical climates in India (Yang *et al*., 2005; Syngenta Corporation). Farming in colder climates is also anticipated since a frost tolerance gene cloned from fish facilitated sugar beet growth at reduced temperatures (Yuancong, 1997). The tropical beet alone is expected to bring significant agronomic and environmental advantages that will deliver output yield comparable to sugarcane for both processing sugar for food and conversion to ethanol for use as a biofuel.

The magnitude of biotechnological advances being made in improving and developing sugar beet varieties will concomitantly intensify its cultivation and the need for effective and environmentally compatible disease and pest control strategies. Therefore, studies aimed at the characterization of genes that are involved in resistance mechanisms are of utmost importance in order to develop alternate control measures that are not based upon environmentally persistent synthetic chemistries.

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